Changes in characteristics of rat skeletal muscle after experimental allergic encephalomyelitis.

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ABSTRACT: Experimental allergic encephalomyelitis (EAE) serves as an animal model for certain neuroinflammatory diseases of the central nervous system, in particular multiple sclerosis (MS). EAE is accompanied by transient weakness or paralysis of hind limbs. We have investigated the effect of partial and transient conduction failure in the central nervous system on skeletal muscle function. At ~2.5 days after development of maximal clinical signs, body and medial gastrocnemius muscle mass were lower (by ~21 and 33%, respectively; $P < 0.05$) in EAE rats compared with controls. Fiber cross-sectional area was lower by 40–50% in all fiber types. Maximal force and power were substantially lower (by 58% and 73%) in EAE rats, as was the force normalized for muscle mass (35%). However, no such weakness was found when lower stimulation frequencies were used. Generation of similar submaximal forces was attributable to a slower relaxation in EAE muscles. This advantage for the EAE muscles was lost during repeated exercise. While fatigability was similar, the difference in relaxation rate between EAE and control disappeared in fatigue. Our data suggest that, as a result of central neuroinflammatory diseases, maximal performance of skeletal muscle is impaired but submaximal performance is relatively well maintained.

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CHANGES IN CHARACTERISTICS OF RAT SKELETAL MUSCLE AFTER EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Muscle weakness[23] and fatigue[30] are important symptoms in patients with multiple sclerosis (MS). The type and extent of neurological deficits in MS vary from attack to attack.[21] During the disease, damage and recovery occur both simultaneously and serially. The therapeutic possibilities for MS are limited, and new approaches to influence the balance between damage and recovery are needed. This is true not only for the demyelinating process, but also for the muscle weakness resulting from the pathological events in the central nervous system.

Abbreviations: CFA, complete Freunds adjuvant; CSA, cross-sectional area; EAE, experimental allergic encephalomyelitis; GM, gastrocnemius medialis; Lo, muscle optimum length; MBP, myelin basic protein; MS, multiple sclerosis

Key words: experimental allergic encephalomyelitis; fatigue; MS; muscle atrophy; muscle weakness

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Apart from the neurologically induced changes in the skeletal muscles, peripheral symptoms may be a consequence of chronically reduced physical activity in MS patients.[19] Relatively small changes in fiber-type composition and a decreased oxidative capacity are indications of adaptation to reduced activity.[16] The lower maximal force–generating capacity of quadriceps muscles of MS patients compared to controls [9] may be related to partial atrophy of the muscle fibers due to reduced activity, but may also be an indication of a lower specific force (force per cross-sectional area) due to reduced activity or to MS-related neurological deficits. However, these findings probably explain only part of the weakness of MS patients. It has been suggested that muscle weakness in MS patients is mainly a result of an impaired voluntary activation of skeletal muscles and that enhanced fatigability results from the higher relative workload of MS patients at the same absolute load, e.g., with carrying their own weight.[9]

Experimental allergic encephalomyelitis (EAE) is an experimental inflammatory demyelinating dis-

ease of the central nervous system, and serves as an animal model to study demyelinating diseases of the central nervous system in general, and MS in particular.[24,25] In the induction phase (days 1–10), animals are immunized with myelin components to induce an anti-myelin immune response.[22,25] In particular, the effector phase of EAE (days 10–18), during which local inflammatory responses cause the actual tissue damage, is relevant for studying the damaging events in MS. Acute actively induced EAE in Lewis rats is characterized by a temporal paresis and paralysis of hind limbs and tail.[22] To our knowledge, no reports are available on the effect of the immune-mediated, transient, and probably partial central conduction failure on the properties of the musculature in EAE rats. In this study, we therefore examined whether changes in skeletal muscle function occur in EAE animals and the nature of any changes. Accordingly, muscle performance and several muscle characteristics were measured in EAE during the recovery phase of the paralytic episode, and compared to those of control rats.

MATERIALS AND METHODS

Experiments were performed using 15 male Lewis rats. Acute EAE was induced in seven rats by a single subcutaneous injection of purified myelin basic protein (MBP) in combination with *Mycobacterium tuberculosis* (37 Hra, Difco, Detroit, MI) and complete Freunds adjuvant (CFA; Difco) under ether anesthesia.[22] Four rats were injected only with CFA (without MBP) and served as control. Another four control rats received no treatment. The EAE and CFA rats were weighed daily and examined for the development of neurological signs. Clinical signs were scored on a scale from 0 to 4: 0, no clinical signs; 0.5, loss of body mass; 1.0, loss of tip of tail reflex; 1.5, complete loss of tail tonus; 2.0 unsteady gait; 2.5, paresis of the hind legs; 3.0, complete paralysis of the hind legs; 3.5, paralysis of the complete lower part of the body; 4.0, death due to EAE.[22] Experiments on muscle function were performed 15–18 days after immunization (or CFA), 2–3 days after the peak of the disease.

The rats were anaesthetized with an initial dose of urethane (1.5 g/kg; i.p). Supplementary injections (0.63 g/kg) were applied if necessary. Medial gastrocnemius (GM) muscles were freed from the surrounding tissue, and the distal tendon with a small part of the calcaneus was connected to a force transducer that was part of an isovelocity measuring system.[6] The proximal origin of the GM was left intact, as was its blood supply. The femur was fixed to the measuring system. Length changes of the GM were induced by a servomotor connected to the lever arm to which the force transducer was mounted. Contractions were induced by electrical stimulation (pulse width, 50 μs) of the sciatic nerve with only the branches leading to the GM left unimpaired. The current amplitude (0.5 mA) was high enough to activate all muscle fibers. To prevent the (reflex) influence of the nervous system, the sciatic nerve was severed as proximal as possible and the distal part was stimulated. The muscle temperature was maintained at 34–36°C with a water-saturated airflow around the muscle, which also kept the muscle moistened. Stimulation and length changes were computer controlled. Force and length signals were digitized (1 or 10 kHz) and stored on disc of the same computer. At the end of the experiments, the GM was excised, weighed, stretched to about optimum length, and frozen in isopentane cooled in liquid nitrogen, and the rats were killed with an overdose of anesthesia.

Rat handling and experiments conformed with the Dutch Research Council's guide for the care and use of laboratory animals.

Protocols. For all muscles, optimum length was first estimated using a few twitch contractions (1 per min). Optimum length (Lo) was then determined with only a few (three to four) tetanic contractions (stimulation frequency, 150 Hz; duration, 150 ms). About 15 min later, the following protocols were started.

Force–Velocity Relation. For the measurements of the force–velocity relation, the muscles were maximally stimulated with a frequency of 400 Hz to ensure maximal activation at all velocities.[10] Contractions were performed during which the muscles were allowed to shorten at a constant velocity (varying from 0–200 mm/s with 10 mm/s intervals). Just before the contraction, the muscle was passively stretched to a length 0.5–1 mm above Lo. Each contraction started with a short isometric phase (duration varying from 10 to 30 ms) during which the force increased to the level that could be sustained during the subsequent shortening at the specific imposed velocity. In this way, the measured force was constant when the muscle passed Lo during shortening.[7] Between contractions, there was at least 2-min rest.

Stimulation Frequency–Force Relation. The influence of stimulation frequency on isometric force production was measured using the following frequencies: 20, between 40 and 160 at 10-Hz intervals, 180, 200, and 250 Hz. The stimulation duration was

150 ms. Recovery time between contractions was at least 1.5 min.

Fatigue. Finally, the muscles underwent a fatigue protocol. This protocol consisted of a series of 20 repeated isometric contractions (stimulation frequency, 150 Hz; duration, 150 ms; 1 contraction every 500 ms).

Analysis of Muscle Samples. Three consecutive cross-sections (10 μm) were cut at mid-level of each GM. One section was stained for connective tissue by incubation in a Sirius Red F3B medium as described by Sant'Ana Pereira et al.[29] The two other cross-sections were stained for myosin adenosinetriphosphatase (mATPase) after alkaline (pH 10.4)[13] and acid (pH 4.7)[1] preincubation.

The cross-sections were digitized and analyzed using a Carl Zeiss KS400 image analysis system with custom-made software. The system consists of a light microscope (Axioskop H; Zeiss, Weesp, The Netherlands), on which a digital camera (Axiocam MRc color version; Zeiss) is mounted. Cross-sections were scanned and divided in an array of 10 $\times$ 10 subsections; each of the subsections was digitized (resolution 800 $\times$ 800 pixels) using a 10$\times$ objective and stored as a JPEG file for further analysis.

The images stained for connective tissue were used to create a mask to identify the same fibers in the serial cross-sections. The cross-sections of GM can be separated in two regions: one containing all fiber types in the proximal part of the muscle, and one containing only type IIX and IIB fibers, located predominantly in the distal part of the muscle.[11] For both regions of each muscle, an area containing about 75 fibers was selected for analysis. Cross-sectional areas (CSA) of the selected fibers were obtained from the mask images. Classification of fiber type occurred by combination of the two mATPase stainings.[29] Six fiber groups were identified: types I, IIA, IIX, and IIB in the proximal region, and types IIX and IIB in the distal region.

Data Analysis. For all isometric contractions, peak force and half-relaxation time were calculated. Half-relaxation time was taken as the time for force to decrease from the maximum to 50% of the maximum at the end of stimulation. To account for differences in muscle mass between groups of rats, the maximal isometric force was also calculated per gram of muscle. The force decrease during the fatigue protocol was expressed relative to the force of the first contraction.

For each muscle, a curve was fitted through the force–velocity data points using the Hill equation. From the fitted curves, maximal shortening velocity, maximal power, and the curvature (a/Fo) were calculated, where Fo is the force of the fitted curve at zero velocity.

The total number of fibers from each type per region (proximal or distal) in all analyzed muscles was determined for the EAE and control groups. From these numbers, the fiber-type distribution was calculated for each region in both groups. The cross-sectional areas of fiber types were averaged for each muscle. Mean ($\pm$ SEM) CSA was then calculated for the EAE and control groups.

Statistics. Differences between mean data $\pm$ standard deviation for the groups were tested using Student's *t*-tests. Differences between the stimulation frequency–force relations of the groups were tested using ANOVA repeated measures ($P < 0.05$). In the case of significant main effects, a Bonferroni post-hoc analysis was performed. A two-way (fiber type and group) ANOVA was performed to test for differences with respect to fiber area ($P < 0.05$).

RESULTS

The experiments with CFA treatment were included in the study as placebo control of possible effects of the anesthesia and treatment procedures. As expected, the animals showed no clinical signs and all data for muscles of CFA-treated rats ($n = 4$) were similar to the control rats without any treatment ($n = 4$). Since there was no indication of an influence of treatment procedures on any parameter, all data for control animals were pooled into one combined group (control; $n = 8$).

All EAE rats showed a lower activity level in their cages due to paralysis of their hind-limb muscles at the peak of the disease. The average maximal score was 2.8 $\pm$ 0.6 (mean $\pm$ SD), which maximum was obtained 14.1 $\pm$ 0.6 days after injection. The measurements of the muscle characteristics were performed 2.5 $\pm$ 1.1 days after the day with the maximal score when the score had decreased to 1.6 $\pm$ 1.0. Only one rat still had a paralysis of the hind-limb muscles on the day of the measurements.

Body and Muscle Mass. There was a significant difference in body mass of the rats between the EAE and control groups (Table 1). This was a result of a significant decrease in body mass of rats in the EAE group from 292 $\pm$ 18 g (10 days after the injection) to 240 $\pm$ 26 g (at the day of the experiment; $n = 8$). For comparison, the body mass of the CFA-treated rats increased significantly from day 10 to day 15

Table 1. Mean (±SD) of rat and medial gastrocnemius muscle characteristics of the EAE and control groups.

	Control (n = 8)	EAE (n = 7)
Body mass (g)	305 ± 31	240 ± 26*
Muscle mass (mg)	783 ± 100	528 ± 110*
Maximal isometric force (F_{max}) (N)	13.3 ± 1.3	5.6 ± 1.6*
F_{max}/muscle mass (N/mg)	17.0 ± 1.2	11.0 ± 3.2*
Half-relaxation time (ms)	39 ± 4	51 ± 7*
Maximal shortening velocity (mm/s)	227 ± 36	242 ± 48
Maximal power (mW)	387 ± 48	106 ± 40*
Force–velocity curvature (a/F_o)	0.64 ± 0.27	0.21 ± 0.04*

EAE, experimental allergic encephalomyelitis.
**Indicates significant difference between the EAE and control groups ($P < 0.05$).*

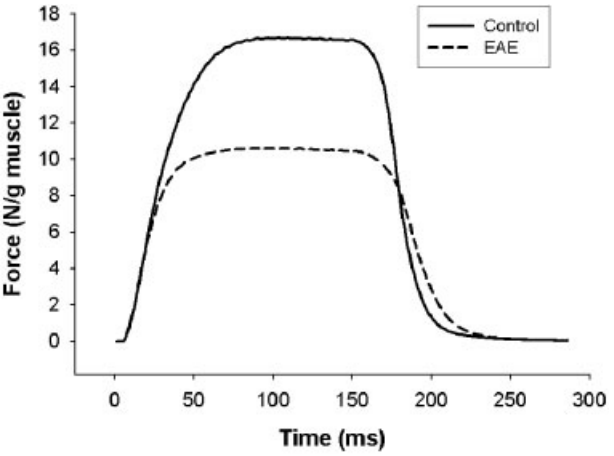

FIGURE 1. Average force traces of a maximal tetanic contraction (150 Hz; 150 ms) of medial gastrocnemius muscles of EAE (dashed line) and control (solid line) rats. The force traces are normalized per gram muscle mass and averaged for seven EAE muscles and eight control muscles.

from 286 ± 25 g to 309 ± 17 g (n = 4). The loss of body mass in the EAE group (by ~21%) was at least partly due to a loss of muscle mass (for the medial gastrocnemius muscle by ~33%; Table 1).

Fiber-Type Distribution and Area. There were freezing artifacts in the cross-sections of one muscle of the combined control group. As a result, analysis was performed in seven muscles of each group. Table 2 shows that the number of fibers analyzed per type and the fiber-type distribution of both the proximal and distal region were not different between the EAE and control groups. The cross-sectional area of all fiber types was ~40–50% lower ($P < 0.05$) in the EAE group than the control group.

Functional Characteristics. ***Maximal Isometric Contractions.*** The maximal isometric force of the EAE group was dramatically lower (by ~58%; $P < 0.05$) than the control group (Table 1). This lower force was not due only to the loss of muscle mass, because the force normalized for muscle mass was also significantly lower (by ~35%; $P < 0.05$). This is illustrated by average force traces of both groups in Figure 1. The figure further shows that the relaxation of the muscles was slower in the EAE group, which is indicated by the significantly longer half-relaxation time (Table 1).

Force–Velocity Characteristics. Group averaged values (± SD) of parameters obtained from the force–velocity curve for each muscle are given in Table 1. The maximal shortening velocity was not

Table 2. Fiber type number, distribution, and cross-sectional area in the proximal and distal regions of medial gastrocnemius muscles of EAE and control rats.†

	Number (n)		Distribution (%)		Cross-sectional area (μm^2)	
Fiber type	Control	EAE	Control	EAE	Control	EAE
Proximal region						
Type I	94	104	17.3	19.0	1,472 ± 134	929 ± 79*
Type IIA	113	110	20.8	20.1	1,336 ± 129	788 ± 80*
Type IIX	253	287	46.5	52.2	1,796 ± 126	1,032 ± 126*
Type IIB	84	46	15.4	8.4	2,347 ± 153	1,254 ± 163*
Distal region						
Type IIX	143	139	25.2	25.5	1,539 ± 137	903 ± 144*
Type IIB	424	406	74.8	74.5	2,624 ± 211	1,223 ± 212*

EAE, experimental allergic encephalomyelitis.
†There were seven rats in both the control and EAE groups.
**Indicates significant difference between the EAE and control groups ($P < 0.05$).*

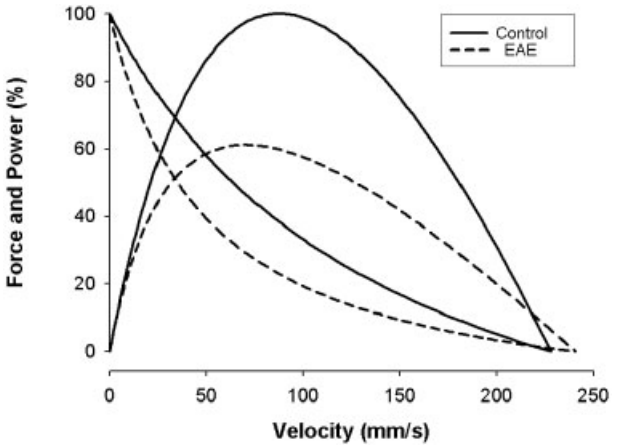

FIGURE 2. Force–velocity and power–velocity relationships for medial gastrocnemius muscles of EAE and control rats. Fitted curves are presented for EAE (dashed lines) and control muscles (solid lines). Force is normalized for the maximal force of each group. Power is normalized for the maximal power of the control group.

significantly different between groups. The maximal power of the EAE muscles was considerably lower (by ~73%; $P < 0.05$). This was not due only to the lower muscle mass and force per muscle mass. The force–velocity relations of the EAE muscles were more curved, leading to an extra reduction of maximal power compared to the control group by ~35% (Fig. 2).

Influence of Stimulation Frequency. Stimulation frequency–force relationships for the EAE and control groups are shown in Figure 3. It can be seen that the stimulation frequency needed to induce maximal isometric force is shifted to lower frequencies for the EAE muscles (from ~150 Hz for the control group to ~80 Hz for the EAE group). As a consequence, in the EAE group, relative high forces were obtained at relative low frequencies of stimulation. The result of this leftward shift is that, although the maximal force of the EAE muscles was only ~65% of that of the control muscles, force generation during submaximal activation was not reduced.

Fatigue. During a series of 20 repeated isometric contractions, a fairly linear decrease in force occurred in both groups to a similar extent (by 32 ± 4% and 30 ± 6% for the EAE and control groups, respectively, at the end of the series). Half-relaxation time increased in both groups, but relatively more in the control group. As a result the difference in half-relaxation time between the EAE and control groups at the beginning of exercise (51 ± 4 ms vs. 40 ± 2 ms) had disappeared at the end of exercise (69 ± 4 ms vs. 69 ± 7 ms).

DISCUSSION

The main results of the present study were: (1) there was a significant loss of body mass, muscle mass, fiber CSA, absolute force and power, and force per muscle mass in the EAE rats; and (2) there was a slower relaxation in the EAE muscles, which had the "advantage" that the stimulation frequency–force relation was shifted to lower frequencies, such that a relative high force was maintained at lower stimulation frequencies, which are used in daily-life activities. This advantage was, however, lost when the muscles fatigued.

Reduction in Mass and Force. To our knowledge, there have been reports of reduction in body mass (e.g., Le Page et al.[18]), but no previous reports of a reduction in muscle mass as a consequence of EAE induction. The loss of body mass started about 10 days after EAE induction, at about the same time as the tip of tail reflex was lost. At the day of measurement, the GM muscle had lost ~33% of its mass (Table 1), indicating that the reduction in body mass was at least in part a consequence of muscle atrophy. It is known that skeletal muscles atrophy very fast when they are not used.[14] The force per unit muscle mass was also considerably lower in the EAE muscles (by ~35%, Table 1, Fig. 1). The occurrence of atrophy in the EAE group was confirmed by the fiber CSA data (Table 2). The differences in fiber CSA (~40–50%) seemed even more pronounced than for muscle mass, but even if this larger difference in

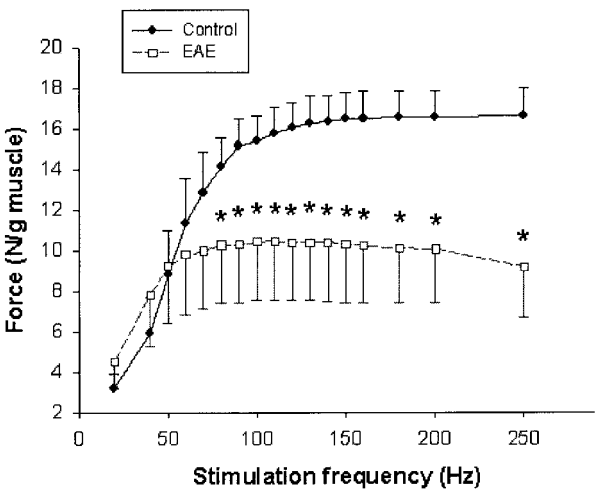

FIGURE 3. Stimulation frequency–force relation for medial gastrocnemius muscles of EAE and control rats. Mean and standard deviations for force per gram muscle are presented for the EAE muscles (n = 7; open squares and dashed line) and control muscles (n = 8; filled circles and solid line). Asterisks indicate significant differences between the groups ($P < 0.01$).

CSA is taken into account, normalized force would still be lower by ~25%.

Similar decreases in specific force have been reported for different forms and degrees of reduced activity.[2,4,15,31] Specific force of soleus motor units was shown to decline as a result of chronic spinal transection in cats.[4] Less drastic models of reduced activity, such as hind-limb unloading[31] and aging,[2] have also resulted in lower specific forces. Several possible mechanisms of the low specific force after reduced activity have been suggested. For example, Kalliainen et al.[15] showed that the specific force deficit could partially be explained by a subpopulation of noncontractile, denervated fibers. Thompson et al.[31] suggested that decreases in myofibrillar protein concentration or number of crossbridges per cross-sectional area are responsible. For human muscle, changes in muscle architecture as a result of atrophy have been held to explain the reduced specific force,[2] although Rutherford and Jones[28] could not demonstrate a relation between fiber pennation and specific force of human quadriceps muscle.

In light of the above-mentioned observations of decreases in muscle mass and specific force as a result of decreased activity, it cannot be concluded that the reduction in force seen in EAE muscles is attributable to EAE. In fact, the reduction in muscle activity alone might explain our result, but a further direct influence of EAE on muscle mass and specific force is possible.

Slowing of Relaxation. We observed longer half-relaxation times in GM of the EAE rats (Table 1, Fig. 1). The rate of muscle relaxation is mainly determined by the kinetics of crossbridge turnover or the rate of removal of calcium ions from troponin as a result of the removal of free calcium from the sarcoplasm by the sarcoplasmic reticulum.[32] The maximal shortening velocity, which reflects the maximum rate of crossbridge turnover,[32] was similar in EAE and control muscles (Table 1, Fig. 2). This suggests that the slow relaxation in EAE muscles is a consequence of slowing of the rate of calcium uptake by the sarcoplasmic reticulum.

It is still unclear which mechanism was responsible for the changes in calcium uptake rate by the sarcoplasmic reticulum but, whatever the mechanism may be, the result of the slowing of calcium uptake may be an advantage for the animals. In EAE, as in MS, it may be that muscle activation is attenuated because of the reduced capacity of axons of motoneurons in brain and spinal cord to conduct the action potential to the peripheral nerves and thus to the muscles. A high firing frequency is needed to fully activate muscle fibers in isometric conditions, but even higher frequencies are necessary for maximal dynamic contractions (maximal power output).[8] However, in daily activities, skeletal muscles are activated submaximally with relatively low frequencies. The advantage of the slowing of relaxation is that muscle force may be relatively high at relatively low frequencies of stimulation (Fig. 3). In the present study, the submaximal forces at low frequencies were similar to those of control muscles despite the lower force per muscle mass and lower muscle mass. The slow relaxation thus enables the EAE rats to sustain daily activities such as eating and drinking. Slow relaxation can, however, also have a negative effect in exercise with fast repeated stretch-shortening contractions when relaxation is too slow to be completed before the muscle is re-stretched for the next contraction. The muscles of the EAE rats were much weaker with regard to maximal forces, and the situation was even worse for maximal power output (Table 1), so that repeated dynamic activity would be difficult for these rats.

Significance for MS. In the present study, the skeletal muscle mass of the hind-limb in EAE rats was reduced by ~30%. Human muscles also show rapid changes in muscle size and function when muscle usage is prevented or reduced. For instance, when muscles were fully immobilized, such as by plaster casting a leg, 40% loss of thigh volume occurred within 6 weeks.[14] In ambulatory MS patients with mean expanded disability status scale (EDSS) ratings of 3.8, however, there was no indication of loss of adductor pollicis muscle mass,[12] and quadriceps muscle mass was reduced maximally by only 11%.[9]

We observed a slowing of relaxation in the muscles of EAE rats (Table 1). In ambulatory MS patients, no signs of slowing in relaxation were found in quadriceps[9] or tibialis anterior muscle.[30] However, for a patient with severe MS, Rice et al.[26] reported a slow relaxation and shift in the stimulation frequency–force relationship in the quadriceps muscle similar to our EAE muscles. A high prevalence of vitamin D deficiency has been reported in MS patients[20] and muscle weakness in vitamin D deficiency may be (partly) attributable to changes in calcium fluxes in skeletal muscle upon activation.[5] Furthermore, vitamin D–depleted rats showed slowing of relaxation,[27] similar to our EAE rats and the severe MS patient,[26] indicating that similar mechanisms may be involved in the changes in muscle function in EAE and MS.

It is clear that the results obtained in EAE rats cannot be translated directly to the situation in all

MS patients. However, our studies clearly show an enormous impact of impaired conduction in the central nervous system on muscle function, which is in accordance with studies after peripheral denervation, and supports the fundamental role of activity as a regulatory signal for muscle contractile properties.[3] Given the fact that in EAE the central conduction failure is only transient, our data encourage us to undertake further studies to preserve muscular activity during EAE, for example, by altering calcium balance. By such approaches, muscle atrophy may be reduced and fatigue partly prevented. Also in MS, certainly at its early stages, neurological deficits are often transient and further studies on the mechanisms of muscle weakness and fatigue are required to investigate whether treatments can be designed, for example, by specific diets or by training,[17] to prevent wasting of muscles.

In conclusion, the present study showed a loss of muscle and body mass starting ~10 days after EAE induction. The loss of muscle mass was accompanied by a reduction in force per muscle mass. Both these changes may be a result of the sudden inactivity of muscles due to the neurological lesion. Two to three days after the time of maximal paralysis, the muscles showed a slowing of relaxation, which shifted the stimulation frequency–force relation to lower frequencies. As a consequence, the submaximal force (at low frequencies) was not lower than control muscles, despite the lower maximal force (only 42%) due to the reduction in muscle mass and the force per muscle mass. This advantage of the EAE muscles was, however, lost after a bout of fatiguing exercise.

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